METABOLISM OF [^{14}C]CHOLESTEROL TO C-20 ISOMERIC [^{14}C]PREGN-5-ENE-3,20-DIOLS IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

Ruben Lozano, Malcolm J. Thompson, James A. Svoboda, William R. Lusby, and Kenneth R. Wilzer, Jr.

Insect and Nematode Hormone Laboratory, ARS, USDA, Beltsville, MD 20705, USA

Corresponding author: Dr. James A. Svoboda, Insect and Nematode Hormone Laboratory, ARS-USDA, Bldg. 467, Room 6, BARC-East, Beltsville, MD 20705, USA
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ABSTRACT

After injection into male and female fifth-instar larvae of Manduca sexta, [^{14}C]cholesterol was converted to C_{21} steroids, [^{14}C]pregn-5-ene-3β,20-diols. These metabolites were isolated from 8-day-old pupae and were identified by TLC, HPLC, and GC-MS as the C-20 isomers of pregnene-3β,20-diol. They also were isolated from male and female meconium fluid (of 16-day-old pupae) following injection of [^{14}C]cholesterol into 14-day-old pupae.

INTRODUCTION

Insects, unlike vertebrates, are incapable of de novo sterol biosynthesis and possess a nutritional requirement for sterols to support normal growth, development, and reproduction (1). Insects do have the ability to metabolize dietary sterols and other steroids in numerous ways, including esterification and hydrolysis, reduction, oxidation, hydroxylation, dealkylation, and conjugation (2). In addition, side-chain cleavage of

sterols, which leads to the formation of vertebrate-type C_{18}, C_{19}, and C_{21} steroid hormones (1), may be a more common metabolic occurrence in insects than previously thought. Whether such steroids retain similar hormonal functions in invertebrates remains to be demonstrated.

From studies of ecdysteroid hormone metabolism in the tobacco hornworm Manduca sexta, we recently demonstrated the conversion of [^{14}C]cholesterol to a triglucoside and a diglucoside of the C_{21} steroid, [^{14}C]pregn-5-ene-3β,20β-diol (3,4). These steroid conjugates were isolated from adult ovaries and eggs of M. sexta that had been injected as 16-day-old female pupae with [^{14}C]cholesterol. The pregnenediol triglucoside was in fact the major metabolite of cholesterol in these eggs, but could not be found in newly emerged larvae. Rather, the steroid glucoside was located on the surface of the eggs, which suggested that the compound might serve a protective role as an antibacterial or antifungal agent or as a feeding deterrent against predators. We now report the conversion of [^{14}C]cholesterol to C-20 isomeric [^{14}C]pregnenediols in another developmental stage of M. sexta, namely in both male and female pupae.

EXPERIMENTAL

Biological Material: M. sexta larvae were reared on an artificial diet. One μCi of [^{14}C]cholesterol in 25 μL of a saline solution containing 3% Tween 80 was injected with a

microsyringe through the dorsal horn of each newly molted fifth (final)-instar larva. Pupal ecdysis occurs 9-11 days later. Eight days after pupal ecdysis, the approximate time of peak titer of molting hormone activity (5) as determined by the house fly assay (6), sexed pupae were collected, weighed, slit several times through the cuticle to enhance solvent penetration, and stored in methanol at -20 C until extraction. Pupae were collected and extracted in batches of 35-65 pupae (about 4.3 g/pupa fresh wt). Newly pupated animals (0-1-day-old) were also collected for extraction and analysis. A short-term labeling experiment was conducted in which 14-day-old M. sexta pupae were injected with [^{14}C]cholesterol through the ventral abdominal segments. After 2-3 days, the last three abdominal segments of the pupal cuticle were removed and the meconium fluid was expressed into a collecting vessel by gentle pressure upon the abdomen. The collected meconium was extracted by the same procedure as that used for whole pupae.

Isolation of Pregnenediol: Whole pupae were homogenized successively in $CHCl_3$/methanol (2:1), methanol, and 70% aqueous methanol (7). The total dried extract was partitioned between hexane and 70% methanol. The 70% methanol phase was dried and the residue was partitioned between n-butanol and H_2O. The butanol phase was dried and the residue was chromatographed on a Florisil column that was eluted with $CHCl_3$ followed by an increasing gradient of ethanol in $CHCl_3$. Fractions were monitored by TLC. Fractions containing pregnenediol, eluted with 2-10% ethanol/$CHCl_3$, were rechromatographed for purification.

Analyses and Instrumentation: Samples were counted in a Beckman LS5801 liquid scintillation system. TLC was done on high-performance silica gel 60 F_{254} plates (Merck) developed in diethyl ether/acetic acid (99:1). Reverse-phase HPLC was performed on a C_{18} column (4.6 x 250 mm, Shandon ODS-Hypersil, 5 µm) eluted with 80% aqueous methanol using a Spectra-Physics SP8700 solvent delivery system; effluent absorbance was monitored at 214 nm with a Waters model 441 detector; effluent was fraction-collected for radioactivity counting. Capillary GC was performed at 210 C on a DB-1 fused silica column (15 m x 0.25 mm, 0.25 µm film, J&W) in a Varian 3700 instrument equipped with a flame ionization detector. Preparative GC analyses were performed on an SPB-1 glass capillary column (30 m x 0.75 mm, 1.0 µm film, Supelco) using a thermal conductivity detector; effluent fractions were trapped for counting. Mass spectra were obtained from a Finnigan model 4500 gas chromatograph-mass spectrometer fitted with a DB-1 fused silica capillary column (30 m x 0.32 mm, 0.25 µm film). GC

temperature was 255 C; EI spectra were collected at 70 eV and a source block temperature of 150 C.

Chemicals: [4-^{14}C]Cholesterol was purchased from Amersham Corp., purified by column chromatography to a radiochemical purity >99% by TLC, and used at a specific activity of 53.7 mCi/mmol. 5α-Pregnane-3α,20β-diol, the 20α- and 20β-isomers of 5α-pregnane-3β,20-diol, and the 20α- and 20β-isomers of pregn-5-ene-3β,20-diol were purchased from Sigma and used for analytical comparison of C-3 and C-20 isomers.

RESULTS

After its injection into newly molted fifth-instar larvae of M. sexta, [^{14}C]cholesterol was metabolized to various radiolabeled products that were isolated and identified from 8-day-old male and female pupae, which resulted in a long-term labeling interval of about 18 days. The bulk of the injected cholesterol was unmetabolized; it accounted for about 96% of the recovered radioactivity. The metabolites were mostly in the form of free, acidic, and conjugated ecdysteroids (<2% of the recovered radioactivity) and cholesterol sulfate (<2%). Much smaller amounts of other radiolabeled compounds (<0.1%), fractionated from the butanol phase partitioned against H_2O, were detected by TLC as being intermediate in polarity between cholesterol and ecdysone. Both sexes of pupae demonstrated similar profiles of their radiolabeled metabolites from cholesterol.

Following further column purification, the unknown material yielded two radiolabeled TLC spots that showed no UV absorbance

at 254 nm and comigrated with authentic 20α (R_f 0.45) and 20β (R_f 0.50) isomers of pregn-5-ene-3β,20-diol. Reverse-phase HPLC of the sample followed by counting of the fractionated effluent produced two radiolabeled peaks that eluted at 6.46 and 8.06 min and coincided with the retention times of authentic 20α- and 20β-pregn-5-ene-3β,20-diol, respectively.

Sample analysis by capillary GC (DB-1 column) similarly produced two major peaks with RRTs (relative to cholesterol) identical with those of the 20α (RRT 0.39) and 20β (RRT 0.37) isomers of pregn-5-ene-3β,20-diol. We demonstrated that the GC peaks were radiolabeled by analyzing the sample on an SPB-1 capillary column with a TC detector. The peaks were trapped from the effluent, counted, and found to contain all of the injected radioactivity. Retention times for authentic 20α- and 20β-isomers of pregnenediol on the SPB-1 column at 210 C were 6.20 and 5.90 min, respectively. The chromatographic characteristics of these compounds from M. sexta were not consistent with those expected of pregn-5-ene-3α,20-diols, based on analytical comparisons of authentic C-3 and C-20 isomers of 5α-pregnane-3,20-diol. We therefore believe the sample pregnenediols to be 3β-steroids.

Analysis of the two radiolabeled GC peaks by GC-MS revealed identical EI mass spectra that were consistent with a pregnenediol structure. Both compounds produced molecular ions

at m/z 318 (55%) and characteristic fragments at m/z 300 (28%, M-H_2O), 285 (13%, M-H_2O-CH_3), and 267 (20%, M-$2H_2O$-CH_3). Mass spectra of authentic C-20 isomers of pregn-5-ene-3β,20-diol were indistinguishable from each other.

In both male and female 8-day-old pupae, the total pregnenediols comprised only 0.02-0.03% of the extracted radioactivity, or about 0.1 μg/g of pupa. In contrast, a far greater amount of cholesterol was converted to free ecdysteroids that were detected at levels of 2.9 μg/g in males and 3.1 μg/g in females (8). The pregn-5-ene-3β,20-diols from male pupae were composed of 36% as the 20α-isomer and 64% as the 20β-isomer. In female pupae, the C-20 α:β composition was 32:68. We have also made a preliminary detection of radiolabeled pregnene-3β,20β-diol in 0-day male pupae that also were injected with [^{14}C]cholesterol as fifth-instar larvae; the suspected pregnenediol accounted for only 0.01% of the total recovered radioactivity at this stage of development.

In a shorter-term labeling study, 14-day-old male and female pupae were injected with [^{14}C]cholesterol; 2-3 days later, just before adult eclosion, meconium fluid (collection of excretory products) was collected for extraction. The C-20 isomers of pregn-5-ene-3β,20-diol, identified by GC-MS, were isolated as radiolabeled metabolites from the meconium of both sexes. The total pregnenediols from male meconium constituted

4.5% of the total extracted radioactivity, while those from female meconium were 3.1%. The C-20 α:β isomeric composition of the pregnenediols from meconium was 80:20 in males and 70:30 in females.

DISCUSSION

With greater attention being directed toward the identification of various C_{18}, C_{19}, and C_{21} steroids in insects, the occurrence of these vertebrate-type steroids in invertebrates can no longer be considered uncommon. These vertebrate steroid hormones have been found in the prothoracic defensive-gland secretion of dytiscid water beetles and shown to be biosynthesized from [^{14}C]cholesterol (9). Several 20-ketopregnanes have been characterized from the defensive anal effluent discharged by carrion beetles of the genus Silpha (10). A number of C_{19} and C_{21} steroids have been identified in the fleshfly, Sarcophaga bullata (11), the locust, Locusta migratoria (12), and the Colorado potato beetle, Leptinotarsa decemlineata (13). Estradiol has been identified in silkworm ovaries (14), and estrogens have been detected in insects representing five different orders (15). Previous studies from our laboratory provided the first definitive proof of the biosynthesis of a C_{21} steroid conjugate from cholesterol in an insect (3,4).

Pregn-5-ene-3β,20β-diol triglucoside was identified as a major metabolite from [^{14}C]cholesterol in adult ovaries and eggs (>600 μg/g) of M. sexta, and it perhaps plays a defensive role as a chemical protectant against predators.

In this report, we provide further evidence for the ability of M. sexta to execute steroid side-chain cleavage by demonstrating the conversion of cholesterol to C_{21} steroids during larval-pupal-adult development. We have detected pregn-5-ene-3β,20β-diol in newly pupated male M. sexta and both 20α- and 20β-isomers in 8-day-old male and female pupae as metabolites of [^{14}C]cholesterol administered to last-instar larvae. The steroids also were identified in the meconium fluid of 16-day-old pupae. M. sexta thus appears capable of pregnenediol biosynthesis during the larval fifth-instar and also during pupal-adult development, at least from 14 to 16 days old. However, the possibility must be considered that microbial symbionts are present within M. sexta larvae or pupae and may be responsible for the C_{21} steroid formation.

The shift from a predominance of the 20β-pregnenediol in 8-day-old pupae to a predominance of the 20α-isomer in 16-day-old meconium may indicate greater synthesis of the 20α-steroid after 8 days of pupal age. Alternatively, there could be synthesis of both C-20 isomers followed by a selective metabolism of the 20β-isomer, possibly glucosylation, to produce

pregnene-3β,20β-diol mono-, di-, or tri-glucosides, although the presence of these metabolites in the pupae could not be established unequivocally. The primary role of pregn-5-ene-3β,20β-diol in female pupae could very well be as a precursor to the steroid triglucoside found in adult ovaries and eventually on the surface of eggs. Additional biosynthesis of this pregnenediol must occur in the adult female to account for the large quantity of steroid triglucoside found on eggs (3,4). However, the simultaneous occurrence of the C-20 isomeric pregnenediols in male pupae may signify additional functions for these C_{21} steroids.

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